

Modelling the growth of lactic acid bacteria at different temperatures

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ABSTRACT

Mathematical models are widely used to predict the shelf life of foods. Lactic acid bacteria (LAB), particularly *Lactobacillus plantarum*, *Weissella viridescens* and *Lactobacillus sakei*, are the main spoilage bacteria of refrigerated, vacuum-packed meat products, stored in modified atmosphere, and their growth determines the shelf life length of these products. The objective of this study was to model the growth of *L. plantarum*, *W. viridescens* and *L. sakei* under different isothermal cultivation conditions and establish secondary models to describe the effect of temperature on the growth parameters of these bacteria. The LAB growth was evaluated in culture medium at temperatures of 4, 8, 12, 16, 20 and 30 °C. The fit of Baranyi and Roberts (BAR) and Gompertz (GO) primary models to the growth curves of LAB was compared by statistical indices, in which the BAR model showed slightly better fits to the experimental data. The BAR growth parameters were used to establish the secondary models, μ_{max} and N_{max} were established for the three LAB. The power model described the influence of temperature on the parameter λ for *L. plantarum*, and other bacteria showed no lag phase. The growth of LAB was strongly influenced by storage temperature and the obtained models allow predicting the growth of these bacteria within the temperature range from 4 to 30 °C.

Key words: predictive microbiology, shelf life, bacterial spoilage.



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INTRODUCTION

Lactic acid bacteria (LAB) are considered the main microorganisms responsible for spoilage of vacuum-packed meat products stored under refrigerated conditions^{1,2}, and some bacteria of the genus *Lactobacillus* are found naturally on meat and meat products, e.g. *Lactobacillus sakei*, *Weissella viridescens*, *Lactobacillus plantarum* and *Lactobacillus curvatus*^{3,4}. The deterioration caused by the LAB is related to the production of metabolites that cause undesirable changes in appearance, texture and flavor of the food, producing unpleasant odors and flavors, besides producing slime on the products' surface^{5,6,7}.

Meat products have an initial concentration of LAB normally around 10 CFU/g and this concentration often reaches a population of 10⁸ to 10⁹ CFU/g in the stationary phase of bacterial growth, exceeding the microbiological limit to the end of the shelf life of refrigerated meat, which is 10⁷ CFU/g^{1,8}. Mathematical modeling can be used to predict the effect of different combinations of time and temperature on the shelf life of meat products. The modeling of microbial growth in culture medium introduces factors that can be controlled more easily than food matrices⁹.

Changes in pH may also be associated with spoilage of meat products. The lactic acid produced by LAB during growth can reduce the pH of the medium where bacteria are growing. Manios et al.¹⁰ found, by means of high performance liquid chromatography analysis that lactic acid production is primarily responsible for the increased acidity of acid emulsions containing pepper, eggplant and fava beans, caused by *L. plantarum* and *L. brevis*.

There is a large amount and variety of predictive models that can describe isothermal microbial growth curves^{11,12}. Two of the several types of models used are the model of Gompertz¹³ and the model of Baranyi and Roberts¹⁴. These primary models are currently applied often in assessing the growth of different microorganisms^{2,15,16,17}.

Secondary models describe microbial behavior according to the variation of intrinsic and extrinsic factors. The influence of temperature on the microbial growth of LAB can be described by some of these models, e.g. linear equation, square root equation¹⁸, Arrhenius equation, power equation and exponential equation.

The objective of this study was to model the growth of *L. plantarum*, *W. viridescens* and *L. sakei* under isothermal cultivation conditions and establish secondary models describing the effect of temperature on growth parameters: duration of the lag phase (λ), maximum specific growth rate (μ_{max}), maximum bacterial population (N_{max}) and time to reach 10⁷ CFU/mL (t_7), while considering the concentration of LAB that defines the shelf life of meat products.

MATERIAL AND METHODS

Bacteria

The strains of *Lactobacillus plantarum* (CCT 0580 ATCC 8014), *Weissella viridescens* (CCT 5843 ATCC 12706) and *Lactobacillus sakei* (CCT 5841 ATCC 15521) were purchased from André Tosello Foundation of Tropical Cultures (Campinas, Brazil) and stored in polypropylene tubes at the temperature of -24 °C in MRS broth (Man, Rogosa and Sharpe) (Difco, Le Pont de Claix, France) containing 20 % (v/v) glycerol for later use.

Inoculum Preparation

For each LAB, the strain frozen was reactivated in MRS broth at 30 °C in an incubator (Dist, Florianópolis, Brazil) for 18 h. After, viable cell count was performed with the plating technique on bilayer MRS agar medium in sterile disposable petri plates, incubated at 30 °C for 48 h. The count was expressed in CFU/mL. In all experiments

performed, the three LAB had the maximum cell concentration of 10^9 CFU/mL, after incubation for 18 h, which was then treated as the inoculum condition for the other experiments.

Growth Conditions

The experiments with pure LAB cultures were carried out in 250 mL flasks with a volume of 160 mL MRS broth and 1% (v/v) inoculum. The initial concentration of experiments was approximately 10^3 CFU/mL and initial pH was 6.0 (pH meter V620, Analion, Ribeirão Preto, Brazil). The flasks were incubated at six different temperatures: 4, 8, 12, 16, 20 and 30 °C, and growth curves were observed until the stationary phase. This procedure was performed for each of the LAB. The experiments were repeated on different days, thus generating duplicate experiments, and each experiment was carried out daily with duplicate Erlenmeyer flasks. The incubation temperature was recorded by a data logger (Testo 174, Lenzkirch, Germany) with records generated every 5 minutes.

The increase in cell concentration was monitored by counting viable cells by the plating method as described above, and also by measuring absorbance (*abs*) in a spectrophotometer (Bel Photonics 1105, Monza, Italy) at a wavelength of 600 nm, at predetermined time intervals, depending on the temperature of incubation. As soon as *abs* measurement began to detect growth, plating was no longer performed on the samples, and a correlation model was used for each LAB, as described below. pH was monitored for each LAB at the six temperatures.

Correlation Model

Equations (1), (2) and (3) represent the correlations between viable cell count (*N*) and absorbance measurements (*abs*), as previously defined, with R^2 values of 0.936, 0.949 and 0.935 for *L. plantarum*, *W. viridescens* and *L. sakei*, respectively.

$$\ln N = 1.715(\ln abs) + 18.82 \quad (1)$$

$$\ln N = 1.274(\ln abs) + 20.08 \quad (2)$$

$$\ln N = 1.478(\ln abs) + 19.79 \quad (3)$$

Primary Growth Models

The primary models of Baranyi and Roberts (BAR) and Gompertz (GO) were selected to describe the growth of LAB at the six incubation temperatures.

The primary model of Baranyi and Roberts¹⁴ is shown in Equations (4) and (5), in which $\ln N(t)$ is the natural logarithm of viable cell count [CFU/mL] at time *t*, *A(t)* is an adjustment function, and the growth parameters are maximum specific growth rate (μ_{max} [1/h]), initial and maximum bacterial population (N_0 [CFU/mL] and N_{max} [CFU/mL], respectively), and physiological state of cells (h_0). The duration of the lag phase (λ [h]) can be obtained by Equation (6).

$$\ln(N(t)) = \ln(N_0) + \mu_{max}A(t) - \ln\left(1 + \frac{e^{\mu_{max}t} - 1}{e^{(\ln(N_{max}) - \ln(N_0))}}\right) \quad (4)$$

$$A(t) = t + \frac{1}{\mu_{max}} \ln\left(e^{(-\mu_{max}t)} + e^{-h_0} - e^{((-\mu_{max}t) - h_0)}\right) \quad (5)$$

$$\lambda = \frac{h_0}{\mu_{max}} \quad (6)$$

The primary model of Gompertz¹³ is shown in Equation (7), in which $\ln N(t)$ is the natural logarithm of viable cell count [CFU/mL] at time t , C is an empirical parameter equivalent to the natural logarithm of initial microbial count, A is the logarithmic amplitude of the population growth, B is the relative maximum growth rate at time M [1/h] and M is the time required to achieve maximum growth rate [h]. The maximum specific growth rate (μ_{max}) and duration of the lag phase (λ) can be obtained by Equations (8) and (9).

$$\ln(N(t)) = C + A \exp\{-\exp[-B(t - M)]\} \quad (7)$$

$$\mu_{max} = \frac{AB}{e} \quad (8)$$

$$\lambda = M - \frac{1}{B} \quad (9)$$

Primary models were fitted to the experimental data using the software MATLAB R2010a, version 7.10 (Mathworks, Natick, USA).

Secondary Models

Five mathematical equations were assessed as secondary models: linear equation, square root equation, Arrhenius equation, power equation and exponential equation, shown in Equations (10) to (14), respectively. All these secondary models are able to describe the dependence of model parameters up to the optimum growth rate (sub-optimal temperature range). In these equations, p is the parameter of interest to be modeled (λ , μ_{max} , N_{max} or t_7 (time to reach 10^7 log CFU/mL), T is the temperature (°C), T_{min} is the theoretical minimal temperature for microbial growth (°C), a and b are empirical parameters.

Secondary models were fitted to the data using the software MATLAB R2010a, version 7.10 (Mathworks, Natick, USA)

$$p = b + aT \quad (10)$$

$$\sqrt{p} = b(T - T_{min}) \quad (11)$$

$$\ln p = a \left(\frac{1}{T} \right) + b \quad (12)$$

$$p = bT^a \quad (13)$$

$$p = a \exp(bT) \quad (14)$$

Statistical Comparison of the Models

To compare the fitting of the primary and secondary models to the experimental data, the following statistical indices were used: coefficient of determination (R^2), mean squared error (MSE) and *accuracy factor*¹⁹.

RESULTS AND DISCUSSION

Primary Models

According to the analysis of statistical indices (*MSE*, R^2 and *accuracy factor*), the two primary models assessed (BAR and GO) presented a good fit to the growth curves of the bacterial species. For *L. plantarum*, *MSE* values ranged from 0.073 to 0.286 and from 0.152 to 0.296; R^2 values ranged from 0.983 to 0.998 and from 0.980 to 0.995, and the values of the *accuracy factor* ranged from 1.012 to 1.030 and from 1.019 to 1.034 for the models of BAR and GO, respectively. For *W. viridescens*, *MSE* values ranged from 0.109 to 0.218 and from 0.050 to 0.207; R^2 values ranged from 0.995 to 0.997 and from 0.992 to 0.998, and the values of the *accuracy factor* ranged from 1.012 to 1.023 and from 1.010 to 1.021 for the models of BAR and GO, respectively. For *L. sakei*, *MSE* values ranged from 0.070 to 0.443 and from 0.091 to 0.411; R^2 values ranged from 0.986 to 0.997 and from 0.987 to 0.997, and the values of the *accuracy factor* ranged from 1.011 to 1.026 and from 1.010 to 1.025 for the models of BAR and GO, respectively.

The values of statistical indices of the BAR model for *L. plantarum*, *W. viridescens* and *L. sakei* were slightly better than the values of the GO model, as previously observed²⁰. Thus, the BAR model was chosen to define the growth parameters of the three LAB (Table 1) and to obtain the secondary models. Figure 1 shows the fits of BAR model to the growth curves. For ease of viewing, each temperature was represented by only one curve for each experiment.

Table 1. Growth parameters μ_{max} (1/h), λ (h) and $\ln N_{max}$ obtained by fitting the BAR model for *L. plantarum*, *W. viridescens* and *L. sakei* at different cultivation temperatures

	<i>L. plantarum</i>			<i>W. viridescens</i>			<i>L. sakei</i>		
	λ	μ_{max}	$\ln N_{max}$	λ	μ_{max}	$\ln N_{max}$	λ	μ_{max}	$\ln N_{max}$
30 °C	0.3	0.65	22.7	0.0	0.78	21.9	0.0	0.78	21.9
20 °C	2.0	0.30	22.5	0.0	0.43	21.9	0.0	0.34	21.9
16 °C	3.7	0.16	21.9	0.0	0.25	21.8	0.0	0.24	21.7
12 °C	12.0	0.06	22.2	0.0	0.16	21.4	0.0	0.17	21.2
8 °C	127.9	0.01	16.9	0.0	0.06	20.9	0.0	0.06	20.2
4 °C	NG*	NG*	NG*	0.0	0.02	19.4	0.0	0.02	19.5

*NG = no growth

Duration of the lag phase and maximum specific growth rate are strongly influenced by temperature of incubation (Table 1 and Figure 1). With decreasing temperature, there is an increase of lag phase only for *L. plantarum*. The other two bacteria did not show adaptation phase in any of the tested temperatures. There was a decrease in the maximum specific growth rate with the decrease in the incubation temperature. This trend was observed by other authors, who also found that growth parameters of LAB are influenced by varying the growth temperature^{1,2,21}.

There was no growth of *L. plantarum* at 4 °C (Table 1). Dalcanton²¹ also found that *L. plantarum* showed no growth at 4 °C for six months, when evaluating the combined effect of temperature, pH, sodium chloride and sodium lactate in the growth of this LAB. Manios et al.¹⁰ studied the spoilage of acidic emulsified spreads containing pepper, eggplant and fava beans, caused by *L. plantarum* and *L. brevis*, and they observed that the LAB showed no growth in the emulsions at 4 °C.

The most important parameters for the analysis of shelf life of meat products are lag phase and maximum specific growth rate²². However, control of maximum population can also lead to increased shelf life of the contaminated products. Cayré et al.²³, when evaluating the effect of storage temperature (0,8 and 15 °C) on the growth of LAB in meat sausages packed in different oxygen permeability, reported an absence of the lag

phase in the growth curves fitted by the modified Gompertz model. Geitenes et al.²² noted the absence of the lag phase while modeling the growth of LAB in sliced cooked and vacuum packaged ham and formed ham samples at 5 °C. The curves were fitted by the modified Gompertz and Logistic models, and the samples already had high counts on the first day of shelf life.

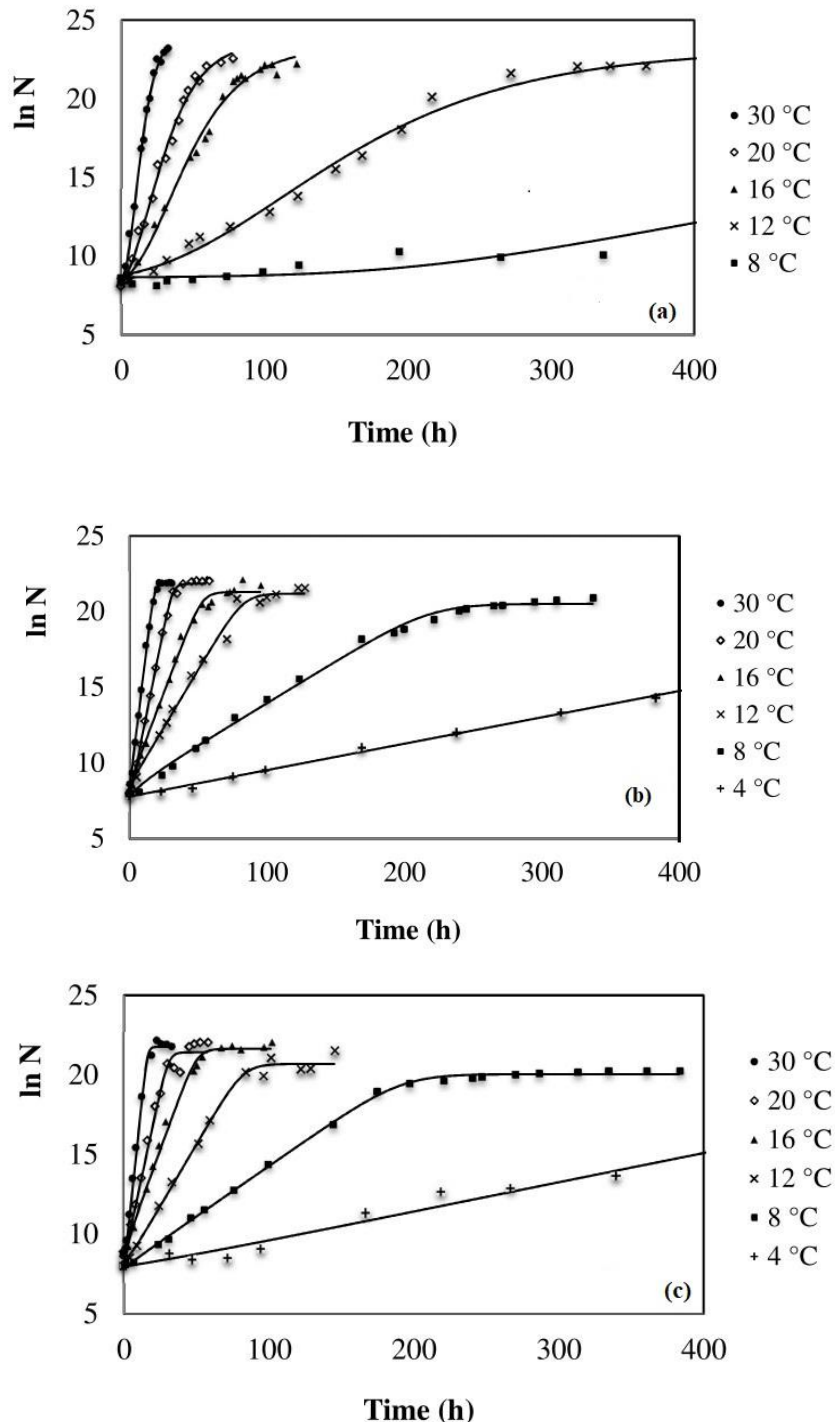


Figure 1. Fitting of the BAR model (solid line) to the experimental data (symbols) of the growth curves of (a) *L. plantarum*, (b) *W. viridescens* and (c) *L. sakei* at different temperatures.

The results in Table 1 and Figure 1 show that it is crucial to maintain incubation temperatures below 8 °C to reduce the growth of LAB. The values of the maximum population at 4 and 8 °C were lower when compared with the other incubation temperatures. This shows that the increase in temperature directly influences the growth of LAB. As meat products are typically stored in a temperature range between 4 °C and 10 °C, it is strongly recommendable that this variable has to be controlled. The increase in the incubation temperature from 8 to 12 °C increases the maximum specific growth rate of LAB by more than 77 %, 60 % and 64 % for *L. plantarum*, *W. viridescens* and *L. sakei*, respectively. These small increases in temperature (ranging from 4 °C to 30 °C) cause changes in all growth parameters, as shown in Figure 1. Thus, in order to control the development and multiplication of spoilage bacteria, such as LAB in meat products that are vacuum-packaged or stored in modified atmosphere, it is crucial to control the temperature in the cold chain.

Secondary Models

The influence of incubation temperature (4 to 30 °C) on growth parameters, obtained by fitting the BAR model, was described by comparing (R^2) of the fits of the Linear, Square Root, Arrhenius, Power and Exponential equations to the experimental data. Table 2 shows the secondary models that best described such influence for all three bacteria.

Table 2. Equations of secondary models that best described the influence of temperature on the growth parameters μ_{max} (1/h), λ (h), N_{max} (CFU/mL) and t_7 (hours) of *L. plantarum*, *W. viridescens* and *L. sakei*.

Microorganism	Parameter	Equation	R ²	Model
<i>L. plantarum</i>	λ	$\lambda = 10^6(T^{-4.517})$	0.989	Power
	μ_{max}	$\sqrt{\mu_{max}} = 0.031T - 0.127$	0.992	Square root
	N_{max}	$\ln N_{max} = -3.166\left(\frac{1}{T}\right) + 3.273$	0.786	Arrhenius
	t_7	$t_7 = 285,335(T^{-3.023})$	0.975	Power
<i>W. viridescens</i>	μ_{max}	$\sqrt{\mu_{max}} = 0.028T + 0.038$	0.993	Square root
	N_{max}	$\ln N_{max} = -0.591\left(\frac{1}{T}\right) + 3.112$	0.991	Arrhenius
	t_7	$t_7 = 9,502(T^{-2.021})$	0.989	Power
<i>L. sakei</i>	μ_{max}	$\sqrt{\mu_{max}} = 0.028T + 0.037$	0.993	Square root
	N_{max}	$\ln N_{max} = -0.581\left(\frac{1}{T}\right) + 3.105$	0.923	Arrhenius
	t_7	$t_7 = 6,159(T^{-1.864})$	0.990	Power

The equations fitted well to the data, resulting in good determination coefficients, as can be seen in Table 2. The growth parameters of *L. plantarum*, *W. viridescens* and *L. sakei* can be predicted using the equations in Table 2 within the temperature range considered in this study (4 °C to 30 °C).

The parameters of microbial growth (duration of the lag phase and maximum specific growth rate) are associated with the shelf life of foods^{16,24,25}. Any change in these growth parameters, caused by variation in the incubation temperature, will interfere in

determining shelf life, or in determining the time needed to reach the LAB concentration of 10^7 CFU/mL (t_7).

The microbiological limit for LAB that has been verified as the end of the shelf life of refrigerated meat products (depending on the organism, the food, and the individual preferences of the consumer) is 10^7 CFU/g^{1,4}. The time needed to reach the LAB concentration of 10^7 CFU/mL (t_7) estimated by the BAR model to the growth curves of *L. plantarum*, *W. viridescens* and *L. sakei* are shown in Table 3. According to the analysis of the values obtained for the t_7 of each LAB, the abuse of temperature contributes to the development and proliferation of LAB, which are the major spoilage bacteria of meat and meat products that are vacuum-packed or stored in modified atmosphere. Again, these results show ones that it is strongly recommendable the control of refrigeration temperature.

Table 3. Time (in hours) needed to reach the LAB concentration of 10^7 CFU/mL estimated by the BAR model to the growth curves of *L. plantarum*, *W. viridescens* and *L. sakei*.

	<i>L. plantarum</i>	<i>W. viridescens</i>	<i>L. sakei</i>
30 °C	13	12	13
20 °C	27	22	21
16 °C	53	31	31
12 °C	140	51	53
8 °C	673	144	146
4 °C	NG*	653	476

*NG = no growth

Spoilage of meat products is analyzed by comparing sensory evaluation with microbial growth. Foods often reach the end of shelf life (7 log CFU/g) showing strong signs of sensory spoilage²⁶. Dalcanton et al.⁴ observed delayed sensory changes of vacuum-packed chopped cooked pork for the three incubation temperatures (4, 10 and 16 °C). They found that spoilage progresses slowly and starts when the cell concentration of *L. plantarum* is above 7 log CFU/g for incubation temperatures of 10 and 16 °C, because at the temperature of 4 °C, the maximum population attained was 5.25 log CFU/g.

pH of LAB

In Figure 2, there was a decrease in pH values, i.e., an increase of lactic acid concentration when the cell concentration of *L. plantarum* reached values of 7 log CFU/mL for the temperatures of 30, 20, 16 and 12 °C, and for 8 °C, cell concentration was 6 log CFU/mL. For *W. viridescens* and *L. sakei*, pH values started to decrease when cell concentration reached values of 8 log CFU/mL.

These differences between decrease in pH and cell concentration reached by LAB are because these bacteria belong to different species, thus producing different results. Similar results were also observed by Ruiz-Capillas et al.²⁷ and Kreyenschmidt et al.²⁸, who found that pH remains constant until the cell concentration of LAB reaches values of approximately 7 log CFU/g. However, in the studies of Vasilopoulos et al.²⁹ and Mataragas et al.³⁰, pH values decreased when the cell concentration of the LAB was 6 log CFU/g and 8 log CFU/g, respectively.

As the pH values of the LAB begin to decrease with high cell concentrations, this factor cannot be used to characterize shelf life, but it indicates the growth stage of the bacteria, as observed by Dalcanton²¹.

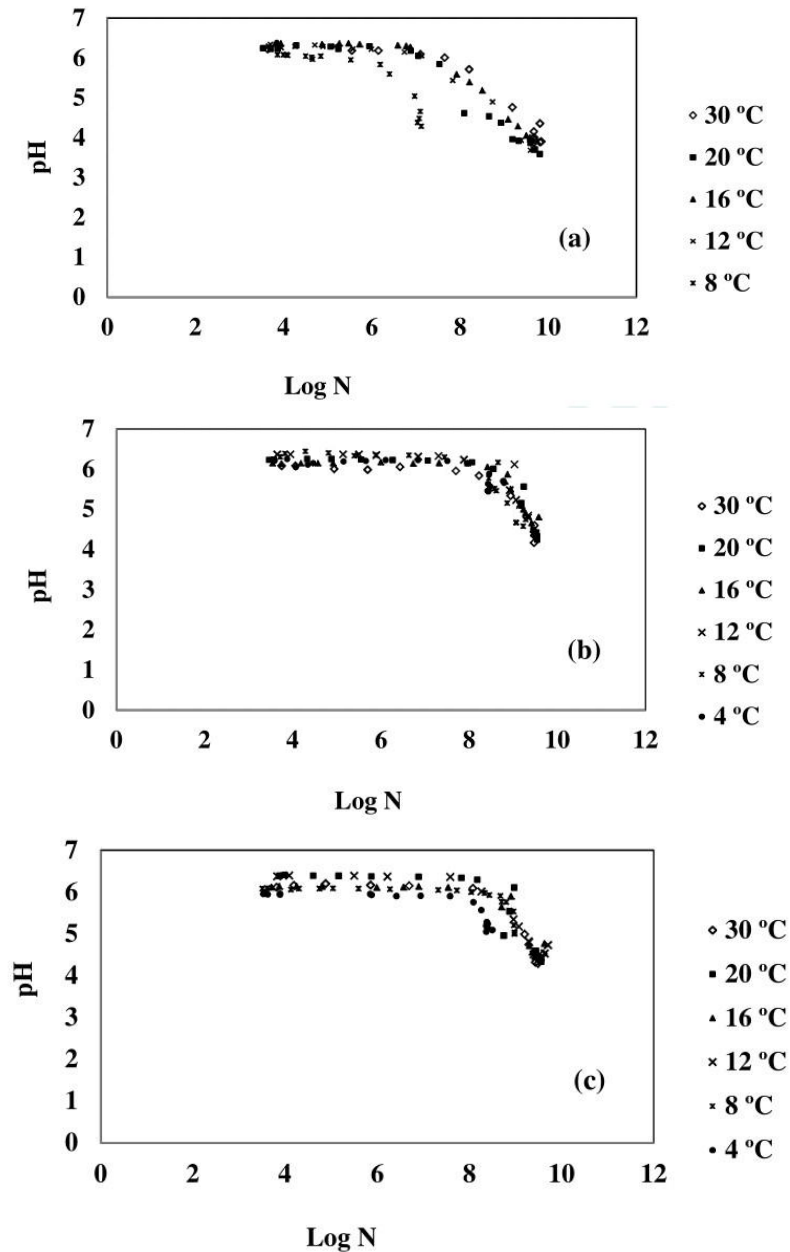


Figure 2. Monitoring of pH changes during growth of (a) *L. plantarum*, (b) *W. viridescens* and (c) *L. sakei* at different incubation temperatures.

CONCLUSION

The both primary models of Baranyi and Roberts and Gompertz showed good fits to the growth curves of *L. plantarum*, *W. viridescens* and *L. sakei*, in which the model of Baranyi and Roberts performed slightly better description of the experimental data than the model of Gompertz.

The secondary models allow predicting the growth of the three LAB within the range from 4 °C to 30 °C. Based on the establishment of both primary and secondary models, it can be concluded that the growth of LAB is strongly influenced by storage temperature, even under refrigerated conditions.

As seen in this study, the abuse of temperature or the temperature fluctuations that can happen along the cold chain can strongly influence the growth of LAB and, consequently, the shelf life of meat products in which LAB are present.

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